AD_____

Award Number: W81XWH-06-1-0345

TITLE: Molecular Mechanisms in Compromised Engothelial Barrier during Breast Cancer Metastasis

PRINCIPAL INVESTIGATOR: Teng-Leong Chew

CONTRACTING ORGANIZATION: Northwestern University
                                                  Evanston, IL 60208-0110

REPORT DATE: March 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                          Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                                          Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE			*Form Approved* *OMB No. 0704-0188*
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**			

1. REPORT DATE 01-03-2007	2. REPORT TYPE Annual	3. DATES COVERED 15 Feb 2006 – 14 Feb 2007
4. TITLE AND SUBTITLE Molecular Mechanisms in Compromised Engothelial Barrier during Breast Cancer Metastasis		5a. CONTRACT NUMBER
		5b. GRANT NUMBER W81XWH-06-1-0345
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) Teng-Leong Chew Email: t-chew@northwestern.edu		5d. PROJECT NUMBER
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Northwestern University Evanston, IL 60208-0110		8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSOR/MONITOR'S ACRONYM(S)
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
   Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT
In this funding period (first of three years), we have fulfilled the scheduled tasks of (1) standardizing the condition for engineering the 3D vasculature network that can consistently produce lumen; (2) generating various viral constructs that can deliver the FRET sensors to 100% of the endothelial cells used in vasculature formation; (3) determining the fluid exchange rate within the 3D collagen matrix that will be critical for any subsequent drug treatment of the endothelial cells in the assay system; (4) co-developing with Improvision a FRET module that is capable of performing 3D ratio-imaging. We presented here, to the best of our knowledge, the first ever three dimensional FRET microscopy data. We have also established that the endothelial cells undergo polarization, similar to those under physiological condition, during in vitro vasculogenesis. Taken together, these developments meet and exceed the scheduled tasks outlined in the statement of work, and complete the establishment of the 3D FRET assay system needed to study the transient modulation of endothelial signaling events by breast cancer cells during transendothelial migration.

15. SUBJECT TERMS
   FRET, signal transduction, metastasis, myosin light chain kinase, vasculogenesis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	7	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Introduction

We seek to study in this project the signaling cascades in the underlying endothelium triggered by metastatic breast cancer cells during diapedesis. We proposed to examine these transient and localized signaling events using a three dimensional (3D) assay with superior spatio-temporal resolution. Specifically, we planned to develop and fully integrate 3D fluorescence resonant energy transfer (FRET) studies into 3D tissue engineering to create a vasculature network capable of dynamic read-out of signaling events. This system will allow us to monitor the regional interaction between cancer cells and the endothelial layer. We will use this system to (i) decipher how the metastatic tumor cells increase vascular permeability by controlling the signals that converge on the contractile machinery, (ii) to explore the potential anti-metastatic effects of obliterating these signaling components along the vascular permeability pathway, thus potentially offering new rationale to use the inhibitors as anti-cancer agents. In this update, the progress of year 1 of the Idea Award will be reported according to the approved statement of work. We have spent the first year of the funding period, as planned, working on improving the basic protocol needed to carry out this innovative assay system. We have made significant progress on multiple fronts, including standardizing protocols to reproducibly generate 3D vascular network *in vitro*, creating multiple viral constructs to deliver the FRET sensors to 100% of the endothelial population, as well as characterizing the fluid dynamics within the 3D gel matrix. We have also collaborated with Improvision, Inc. to develop a software module capable of performing 3D ratio-imaging so that we can analyze and display the 3D FRET data. The progress thus far have met, and in some cases exceeded, the timeline originally planned in the statement of work. We do not see the need for any significant alteration of the plan at this point.

A. Development planned in Statement of Work

A. 1. Generation of viral vector to achieve 100% endothelial cell transfection rate (Months 1-4)

In order to ensure that 100% of the endothelial cells forming the vasculature are transfected, we have initially generated adenovirus constructs expressing the FRET biosensor. Unfortunately, the endothelial cells did not tolerate the infection by adenovirus particularly well in our hands. While the biosensor expression level was very high, the endothelial cells displayed rather sickly phenotype and did not form the vasculature network *in vitro* as extensively as uninfected control cells. It was certainly possible that the high level of biosensor expression might have contributed to the aberrant phenotype and the attenuated ability to undergo vasculogenesis. We have however tested endothelial cells transiently transfected with CMV promoter-driven expression vectors of these biosensors; and despite the comparably high level of protein expression, these cells behaved normally. This result thus ruled out the notion that high level of biosensor expression was detrimental to the process of *in vitro* vasculogenesis. To ensure that we achieve 100% transfection efficiency of the endothelial cells, we have generated Lentivirus constructs that will allow us to select for stable transfectants. Endothelial cells tolerated the Lentivirus infection significantly better than Adenovirus, and underwent extensive vasculogenesis *in vitro*. We are now in the process of propagating our stable lines of endothelial cells expressing the biosensors.
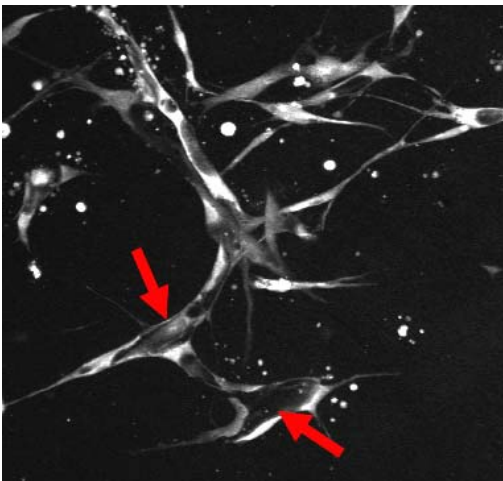

Figure 1: A confocal optical section through the 3D collagen gel 24 hours post-assembly, showing the increased vacuole formation (arrows) induced by phorbol ester. These vacuoles, as previously reported, will eventually fuse to form continuous lumen in the vasculature.

A. 2. Establishing vasculature with lumen (Months 1-9)

The formation of endothelial tubes or lumen in our engineered 3D vasculature network is a vitally important step to the success of the proposed project. We have encountered numerous problems during the initial phase – namely, (a) excessive contraction of the collagen gel by the endothelial cell population as they coalesced to form vessels; (b) inconsistent formation of lumen in the vessels; (c) rapid regression of the formed vasculature. After testing numerous conditions to generate 3D vasculature *in vitro*, we have now established a protocol whereby extensive capillary network with lumen can be reproducibly generated within three dimensional collagen gel. Using time-lapse multi-photon confocal microscopy, Davis et al. (2, 4) have shown that endothelial cell lumenization is a continual process that begins with the formation of pinocytic vacuoles. The fusion of the endothelial vacuoles then drives the formation of lumen. This finding has been observed not only *in vitro* by other groups, several investigators have also observed that many organisms appear to develop endothelial tubes using similar mechanisms (reviewed in (2). In the first nine months of the funded period, we geared our effort toward duplicating this process of lumenization in our vasculature network. We report here that we have developed the protocol with which we can reproducibly generate lumen in the engineered vessels in approximately 3-4 days. By adding matrigel to the collagen mixture we are now able to induce the onset of vasculogenesis within 24 hours after gel assembly. We

also identified laminin-10 and phorbol ester to be of critical importance. Laminin-10 adds critical stability to the vasculature and greatly reduced the extent to which the endothelial contract the gel. As shown in figure 1, the addition of phorbol ester induced pinocytic vacuole formation which subsequently led to the fusion of these vacuoles into continuous lumen, as previously reported (2, 4).

A. 3.   Forming vasculature with endothelial cells expressing FRET-based sensors (Months 9-12)

All the FRET sensors used in this study are robust signaling molecules. It is our main concern that over-expressing these signaling molecules may obliterate the ability of the endothelial cells to form blood vessels. We are delighted to report that over-expression of myosin light chain kinase (MLCK) FRET sensor in the endothelial cells did not hamper their ability to form vasculature network in our *in vitro* system (please refer to section B.2. to see the 3D rendering of *in vitro* vessel expressing the MLCK FRET sensor). The Rho FRET biosensor, on the other hand, was toxic to the cells, causing massive cell death in culture. As proposed in the grant, we would need to put the expression of the Rho FRET sensor under the control of Tet-on promoter system. To that end, we have obtained a Rho FRET sensor whose expression is controlled by a Tet-on promoter through the generosity of Dr. Klaus Hahn at University of North Carolina at Chapel Hill (5, 6). We will perform experiments to determine if inducing expression of Rho after the vasculature formation will eliminate the negative impact on the endothelial cells and will also allow us the necessary window of time to image the transient signaling events triggered by the invading tumor cells.

Fluid exchange Rate of 3D Collagen Gel (Month 15)

One of the key concerns about the 3D collagen gel is the rate of liquid and gaseous exchange in the deepest portion of the gel, as a slow exchange rate will greatly affect the endothelial cells in the 3D gel. The fluid exchange rate is also critical for subsequent experiments involving the addition of inhibitors against Rho Kinase and MLCK. To confirm that liquid medium can exchange freely within the 3D gel, we assemble the gel within a porous ring that is mounted on a cell culture dish with coverslip bottom (as shown in figure 2, panel A). The Cell Tracer green dye (Invitrogen) was then added to the medium. Optical sections were then obtained using the Zeiss LSM 510 laser scanning confocal. As shown in figure 2B, the cell tracer dye readily penetrated the gel and stained the cells within 5 minutes, even to the very bottom of the gel, where all of our 3D FRET imaging will be performed. There is a decreasing gradient of intensity from the bottom of the gel, a direct result of photobleaching due to repeated laser scanning on the same area. We do not anticipate the photobleaching to be any problem for 3D FRET as the FRET microscopy will be performed on spinning disc confocal which is a lot more sensitive and thus requires less excitation light than the Zeiss laser scanning microscope (please see figure 4). This result establishes the approximate rate at which any added pharmacological agent can penetrate the 3D collagen gel and reach the endothelial cells to exert its effect, a critical step in the second aim wherein we will be testing the effect of statins in attenuating the ability of breast cancer to induce signaling events that may directly induce endothelial cell contraction. In this case, we are ahead of the planned schedule in accomplishing this task.
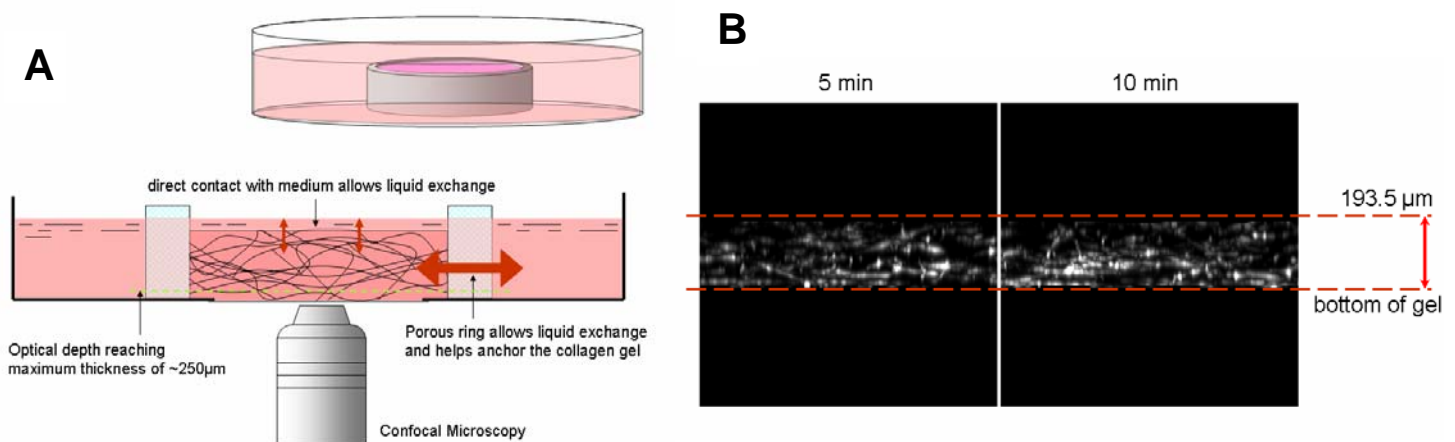

Engineering vasculature network in 3D collagen gel

Figure 2: A. Schematic diagram of the imaging chamber with vasculature assembled within a 3D collagen gel within a porous ring. The pink environment denotes the cell culture medium which can penetrate the porous ring for rapid fluid exchange. Squiggly lines represent the engineered blood vessels. **B.** Orthogonal (side) view of the vasculature gel 193.5 μm into the 3D gel from the coverslip. Z-stacks were taken 5 min and 10 min after green Cell Tracer was added to the culture medium. As shown, the tracer dye penetrated and stained the cells embedded in the deepest portion of the gel in 5 min. Maximal staining was reached in 10 min.

B. Other Important Progress Not Listed in the Original State of Work
B. 1.   Polarization of Endothelial Cells

To determine if the endothelial cells that form the vasculature network within the 3D collagen matrix are polarized, we characterized the pattern of matrix protein synthesized *de novo* by the vessels that have undergone lumenization. If the cells are polarized, they should exhibit a clear luminal and basal polarity that can be easily delineated by the orientation of *de novo* synthesis and depositing of extracellular matrix protein. To achieve this, we used an antibody that recognizes the α4 laminin G domain (3), 2A3 (generous gift from Dr. Jonathan Jones, Northwestern University) to characterize the pattern of α4 laminin expression by the endothelial cells that have successfully formed the lumenized vasculature network. The α4 laminin subunit is a component of endothelial cell basement membrane (3), but is not part of the collagen/matrigel mixture we use to assembly the 3D matrix. As shown in figure 3, endothelial cells (stained green with Cell Tracer cytoplasmic dye) deposited α4 laminin (red) on the peripheral area of the vessel tube but the lumenal space is relatively cleared of synthesized α4 laminin, indicating that the endothelial cells that form the *in vitro* vasculature have polarized, as would the endothelium under physiological condition.
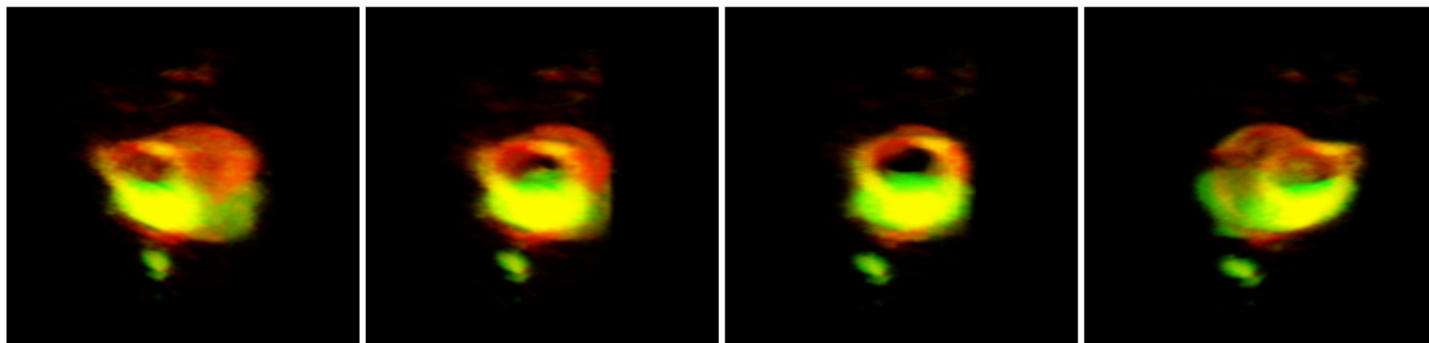

Figure 3: 3D rendering of a vessel segment generated in the collagen gel. The length of the vessel is optically truncated by confocal microscope during image acquisition to highlight the architecture of the lumen. The endothelial cells were pre-stained with Cell Tracer (green), a cytoplasmic marker. 4 days after gel assembly at which time vasculogenesis had occurred, the gel was fixed. 20 μm slices were sectioned on a cryostat and stained with anti-α4 laminin antibody (red). The panels show the α4 laminin expression pattern relative to the lumen as viewed from four different angles, indicating that the endothelial cells have undergo polarization, with almost all the newly synthesized matrix protein being deposited at the basal surface rather than the lumenal surface.

B. 2.   3D FRET ratio-imaging software

In collaboration with the Northwestern University Cell Imaging Facility (of which the principal investigator of this grant is the director), Improvision Inc. has developed a 3D FRET module that has now become part of the standard software package for Volocity (version 4.0.1 onward). This FRET module allows us to perform three-dimensional ratio-imaging, a critical step to display and analyze our 3D FRET data. As shown in figure 4, vasculature formed by endothelial cells expressing the MLCK FRET sensor can be imaged live using our spinning disc confocal microscope, and the ratio data analyzed using intensity modulated display by the Volocity software. It is important to point out that, to the best of our knowledge, these are the first 3D FRET microscopy data generated. We can also use this software module to display this 3D ratio images as a function of time – which is the ultimate goal of this proposed work. We now have the needed temporal and spatial resolution and all the technical means to dynamically characterize the signaling events in any portion of the *in vitro* vasculature that may be triggered by the mestatic breast cancer cells as they undergo transendothelial migration.
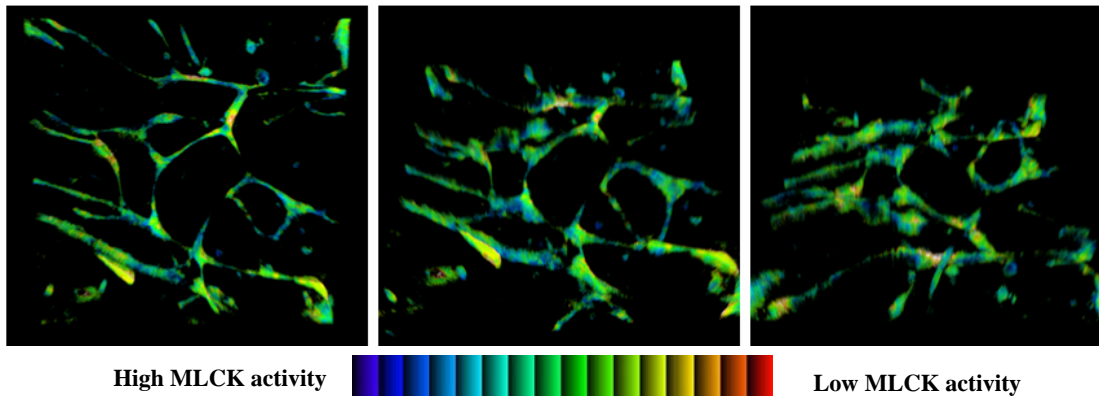

High MLCK activity   **Low MLCK activity**

Figure 4: 3D ratio images of vasculature expressing the MLCK FRET sensors, viewed at various angles, were generated by the Volocity software. Colors denote the relative MLCK activity according to the ratio bar. Ratio images are generated by intensity-modulated display (IMD) as described earlier. (1)

Key Research Accomplishments

- Generation of viral vectors to deliver the FRET sensor constructs to 100% of the endothelial cells used to generate the 3D vasculature

- Standardization of the condition for endothelial cells to reproducibly establish vasculature with lumen in 3D matrix

- Standardization of the condition for endothelial cells expressing the FRET biosensors to establish lumenized 3D vasculature

- Characterization of the fluid exchange rate of the 3D matrix for subsequent drug treatment experiments

- Development of 3D FRET ratio-imaging software module with Improvision, Inc.

- Performing the first 3D FRET confocal microscopy

- Confirmation of the polarization of endothelial cells in the engineered vasculature

Reportable Outcomes

- 3D FRET and ratio-imaging modules now incorporated in Volocity software (Improvision) version 4.0.1 and onward.

Conclusion

We have successfully accomplished all the tasks in the approved statement of work scheduled for the first year of the 3-year funding period. These accomplishments, taken together, demonstrate that we have completely standardized the 3D assay system with which we can now dynamically monitor, with extremely high spatiotemporal resolution, the transient and *in situ* signaling events within the vasculature network triggered by invading breast cancer cells. We are now fully equipped and on schedule to carry out the experimental phase of the proposed project and tackle the first specific aim: *to characterize the signal transduction in endothelial cell in 3D matrix during tumor invasion.*

In this first year, the most critical advancement we have made is the development of the methodology and software module to perform three-dimensional FRET. This microscopy technique will prove to be a powerful improvement not only for those working in breast cancer, but for any investigators who need to expand their FRET imaging repertoire to study dynamic signaling, protein processing, protein-protein interactions as well as protein conformational changes in three dimension either in single cells or in thicker tissue specimens. It also marks the successful combination of advance microscopy technique with *in vitro* tissue engineering.

References

1.   **Chew, T. L., W. A. Wolf, P. J. Gallagher, F. Matsumura, and R. L. Chisholm.** 2002. A fluorescent resonant energy transfer-based biosensor reveals transient and regional myosin light chain kinase activation in lamella and cleavage furrows. J Cell Biol **156:**543-53.

2.   **Davis, G. E., and K. J. Bayless.** 2003. An integrin and Rho GTPase-dependent pinocytic vacuole mechanism controls capillary lumen formation in collagen and fibrin matrices. Microcirculation **10:**27-44.

3.   **Gonzales, M., B. Weksler, D. Tsuruta, R. D. Goldman, K. J. Yoon, S. B. Hopkinson, F. W. Flitney, and J. C. Jones.** 2001. Structure and function of a vimentin-associated matrix adhesion in endothelial cells. Mol Biol Cell **12:**85-100.

4.   **Kamei, M., W. B. Saunders, K. J. Bayless, L. Dye, G. E. Davis, and B. M. Weinstein.** 2006. Endothelial tubes assemble from intracellular vacuoles in vivo. Nature **442:**453-6.

5.   **Pertz, O., L. Hodgson, R. L. Klemke, and K. M. Hahn.** 2006. Spatiotemporal dynamics of RhoA activity in migrating cells. Nature **440:**1069-72.

6.   **Wong, K., O. Pertz, K. Hahn, and H. Bourne.** 2006. Neutrophil polarization: spatiotemporal dynamics of RhoA activity support a self-organizing mechanism. Proc Natl Acad Sci U S A **103:**3639-44.